SUPPLEMENTARY MATERIAL

Production of ethanol, lipid and lactic acid from mixed agrowastes hydrolysate

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Abstract

To combat the stress on single agro-residue and overcome the problem of seasonal availability, it is beneficial to use mixture of lignocellulosic biomasses. In the present study, efforts were made to use mixed lignocellulosic biomass for the production of bioethanol, along with microbial lipids and lactic acid. Upon enzymatic hydrolysis of mixed biomass at varied proportions, it was observed that mixture of paddy straw and jute in the ratio 3:1 resulted in best sugar yield (41.50g/L) at 10% substrate loading. Ethanolic fermentation of mixed substrate hydrolysate by thermotolerant yeast, *Saccharomyces cerevisiae* JRC6 resulted in 8.39 g/L of ethanol. To maintain sustainability and economic impact, oleaginous yeast (*Trichosporon mycotoxinivorans* S2) and lactic acid bacteria (*Lactobacillus plantarum* LP-9) were used for lipid (14.5 g/L) and lactic acid production (11.08g/L), respectively. Therefore, this study explored the potential of mixed lignocellulosic biomass to be exploited for production of various value-added products.

Keywords: Oleaginous yeast; Single Cell Oils; Jute; Mesta; Bioethanol; Lactic Acid Bacteria

Contents

List of Figures

List of tables

Experimental

Raw materials and chemicals

Jute (leftover stalks after fibre extraction) and mesta biomass was procured from ICAR-National Institute of Natural Fibre Engineering and Technology, Kolkata, India. Paddy straw was collected from farms of ICAR-Indian Agricultural Research Institute, New Delhi, India. The biomass was dried in the oven at 60°C for 72 h, mechanically shredded to 0.5–1.0 cm, and stored in airtight containers at room temperature for further analysis. Stock solution of penicillin (10,000 U) and streptomycin (10 mg/mL) was purchased from Himedia Labs, India. All other chemicals and solvents used in the study were purchased from Sisco Research Laboratory, Mumbai, India.

Enzymes

Commercial cellulase (Cat No. C2730, Sigma, USA) and beta-glucosidase enzyme (Cat No. C6105, Sigma, USA) used for saccharification were purchased from Sigma-Aldrich, USA.

Microorganisms

The thermotolerant yeast *Saccharomyces cerevisiae* JRC6 (accession number KX668410), *Trichosporon mycotoxinivorans* S2 (accession number MK752668) and *Lactobacillus plantarum* LP-9 (accession number MT008062), procured from Division of Microbiology, IARI, New Delhi for the fermentation experiments. The yeast cultures i.e., *S. cerevisiae* and *T. mycotoxinivorans* were maintained and grown on MGYP (malt extract 3.00 g L^{-1}, glucose 10.00 g L^{-1}, yeast extract 3.00 g L^{-1}, peptone 5.00 g L^{-1}) at 30°C for 48 h. Cultures were stored at 4°C on MGYP slants and subcultured periodically whereas bacteria *L. plantarum* was grown on MRS broth (proteose peptone 10.00 g L^{-1}, peptone b 10.00 g L^{-1}, yeast extract 5.00 g L^{-1}, dextrose (glucose) 20.00 g L^{-1}, tween 80 (polysorbate 80) 1.00 g L^{-1}, ammonium citrate 2.00 g L^{-1}, sodium acetate 5.00 g L^{-1}, magnesium sulphate 0.10 g L^{-1} , manganese sulphate 0.05 g L^{-1}, dipotassium hydrogen phosphate 2.00 g L^{-1}) at 37°C for 48-72 h and subcultured on MRS agar slants with 1% $CaCO_3$ and stored at 4 °C.

Saccharification of pretreated mixed biomass (Paddy straw: jute/mesta)

All the three biomasses (Jute, mesta and paddy straw) were chopped (1 cm pieces) and pretreated with alkali (2% NaOH v/v) separately in the Erlenmeyer flasks with the substrate loading of 20% by autoclaving at 121 °C for 20 min (hot treatment). It was followed by two washings with distilled water in the same ratio to bring down the pH and the biomass was

vacuum filtered. The washed biomass was then dried, grounded to 1mm particle size and used as the substrate for saccharification.

Alkali pretreated jute and mesta were used individually or in combination with pretreated paddy straw in different combinations for enzymatic saccharification using commercial enzymes. Enzymatic hydrolysis of alkali pre-treated paddy straw, jute and mesta biomass was carried out with substrate loading of 5 and 10 % (w/v) at different ratios (Table S1) in citrate buffer (pH 4.8) with the addition of cellulase enzyme (25 FPU/gds), β-glucosidase enzyme (15 IU/gds), 200ul of sodium azide/ antibiotic solution of penicillin and streptomycin (100X) in 100 mL saccharification tubes. These tubes were incubated in shaking water bath (150rpm) at 50°C for 72 h for enzymatic hydrolysis. Aliquots were withdrawn periodically at 24, 48 and 72 h from the tubes. To remove unhydrolysed residues, samples were filtered and centrifuged at 3000g for 10. The 3,5-dinitrosalicylic acid method was used to determine the reducing sugar content of the supernatant (Miller, 1959). Total sugar released within 72 h were expressed in g/L. All the experiments were performed with 3 replicates and their mean values tabulated. All the data obtained from the experiments were statistically analysed using the Three-Way ANOVA through GRAPHPAD PRISM 8.0 program (Microsoft Excel package, USA). Statistical significance was performed at $P = 0.05$ level, where means of three way interactions was found non-significant However, two-way interaction between substrate loading and substrate ratio, and substrate loading and time were found significant at 0.05% confidence interval.

Alcoholic fermentation of saccharified hydrolysate

The saccharified hydrolysate of alkali pretreated mixed biomass (Paddy straw: Jute/ Mesta) was used for production of ethanol using thermotolerant yeast *S. cerevisiae* JRC6 through submerged fermentation. Inoculum was prepared by growing yeast culture in MGYP medium (24 h old culture) and added at the rate of 10% (v/v) inoculum to the flasks containing saccharified hydrolysate. Fermentation was carried out at 30°C in an incubator for 72 h under stationary conditions. Samples were collected aseptically at interval of 12 h during fermentation process, centrifuged at 10,000 rpm for 10 min and supernatant was collected, filtered and analysed for sugar consumed and ethanol produced by HPLC method.

Utilization of saccharified hydrolysate for lipid production

During ethanolic fermentation with *S. cerevisiae* JRC6 only glucose was utilised and xylose remained unused. Therefore, *T. mycotoxinivorons* S2 yeast which could utilise both hexose and

pentose sugars was used for lipid production using saccharified hydrolysate left after ethanolic fermentation. The hydrolysate was supplemented with yeast extract (0.5g/L), peptone (1.8 g/L) and $MgSO_4$ (0.4 g/L), and inoculated with *T. mycotoxinivorons* S2 yeast @ 10% (v/v) and incubated at 30 °C in an incubator shaker with rotation speed of 120 rpm for 7 days.

Lipid extraction and quantification

After incubation, the hydrolysate was centrifuged at 8000 rpm for 10 min and the biomass pellet was macerated with liquid nitrogen using mortar pestle. Further, the biomass was suspended in distilled water and sonicated under ice-cold condition at 40 pulses (5 times) for 3 min using an ultrasonic homogeniser (Model 3000, Biologics Inc. USA). For lipid separation, solvent mixture of chloroform: methanol in ratio 2:1 was added to the homogenised biomass and was kept undisturbed for about 30 minutes (Poli et al. 2014). After separation of layers, the upper phase was removed without disturbing the interface whereas the lower chloroform layer containing lipids was collected into a pre-weighed container and was kept in oven at 40˚C for solvent evaporation followed by gravimetric estimation of lipid content and lipid yield was expressed as g of lipid L^{-1} of hydrolysate (Kilcawley, 2002).

Fermentation using Lactic acid bacteria Lactobacillus plantarum LP-9

The sugar rich saccharified hydrolysate of mixed substrates was also used for lactic acid production through submerged fermentation using 24 h old culture of *Lactobacillus plantarum* LP-9 grown on MRS medium. The saccharified hydrolysate was supplemented with MRS media (without glucose) and calcium carbonate (2% w/v) to maintain the desirable pH. This fortified hydrolysate was inoculated with 10% (v/v) inoculum of *L. plantarum* LP-9 (Sharma et al. 2021). The organism is microaerophilic; therefore, fermentation was carried out under static condition at 36 °C for 72 h and aliquots were withdrawn aseptically after 24, 48 and 72 h. To estimate residual sugar and quantification of lactic acid samples were centrifuged at 1000 rpm for 10 min and supernatant was analysed by HPLC.

Quantification of sugars, ethanol and lactic acid by HPLC

Presence of sugars, ethanol and lactic acid in the fermented samples were analysed using high performance liquid chromatography (HPLC) Waters 515 (Waters Corporation, Milford, MA, USA) equipped with a Waters 2414 refractive index (RI) and photodiode array (PDA) detector. Sugars and ethanol were detected by RI detector (Glucose RT 8.9 min, Xylose RT 9.6 min, and Arabinose 10.5 min, Ethanol RT 20.3 min) and lactic acid (RT 11.6 min) was detected by PDA

detector. The column Aminex HPX-87H was operated with 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 ml/min. The oven temperature was kept at 60 °C.

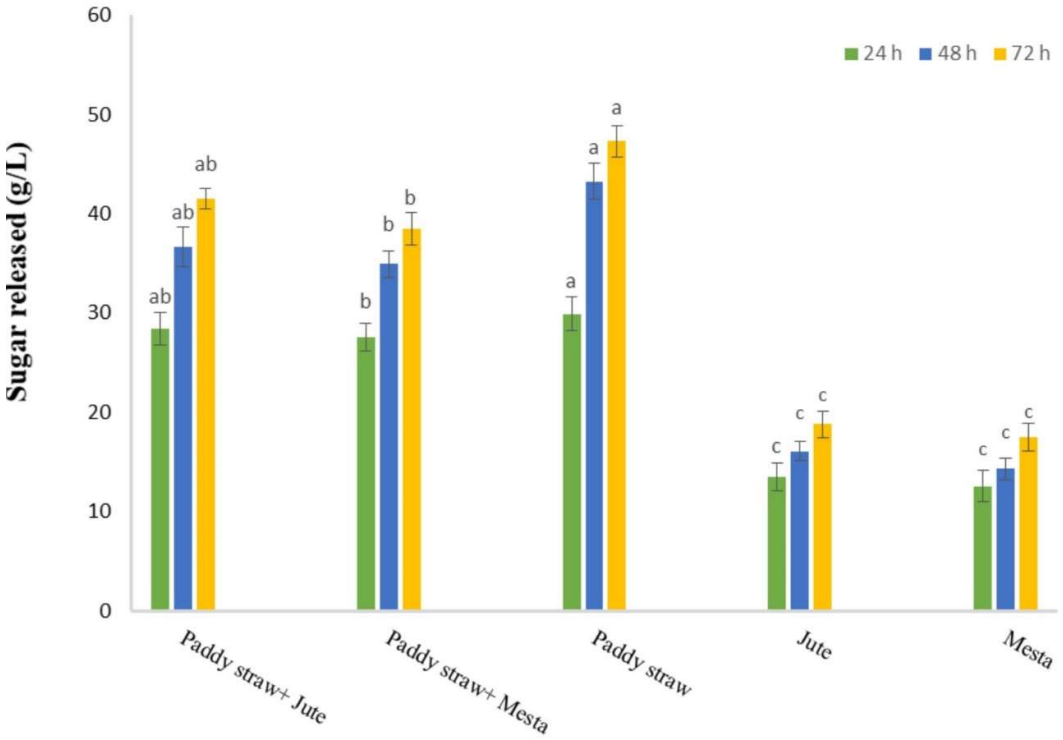

Figure S1. Total reducing sugar released (g/L) after enzymatic saccharification of pretreated individual and mixed biomass in 3:1 ratio indicates highest amount of sugar was released in 72 h of saccharification

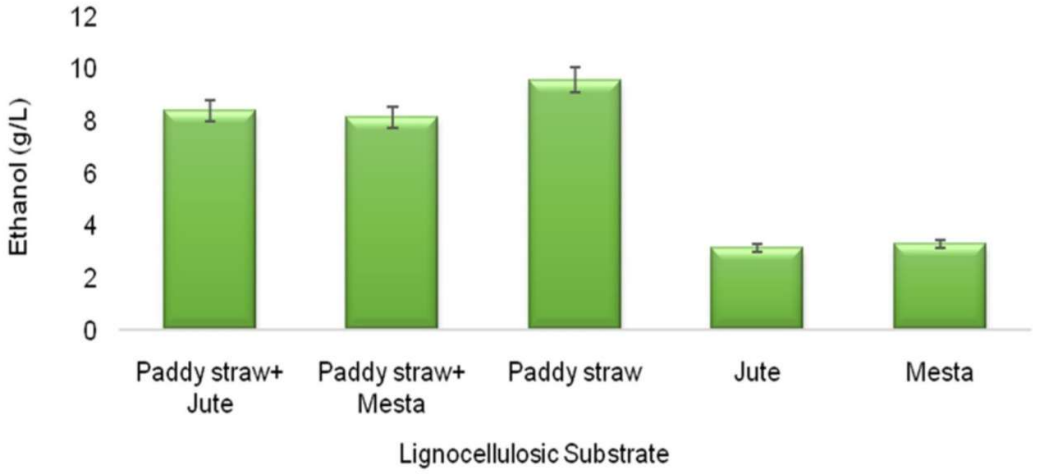

Figure S2. Ethanol production (g/L) from saccharified individual and mixed substrate using *S. cerevisiae* JRC6. Error bars represents SEM.

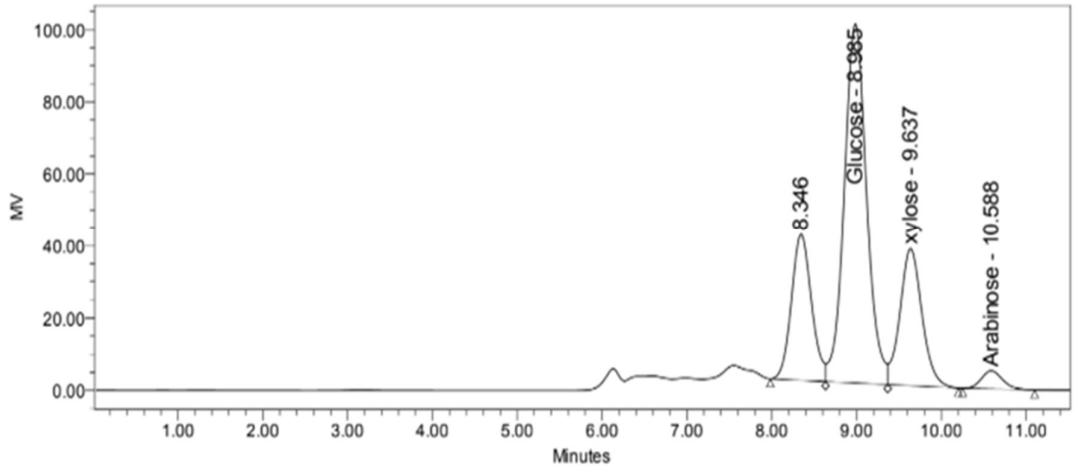

Figure S3. HPLC chromatogram showing peaks of sugar released after 72 h saccharification from mixed biomass (Paddy straw: Jute) at 3:1 ratio.

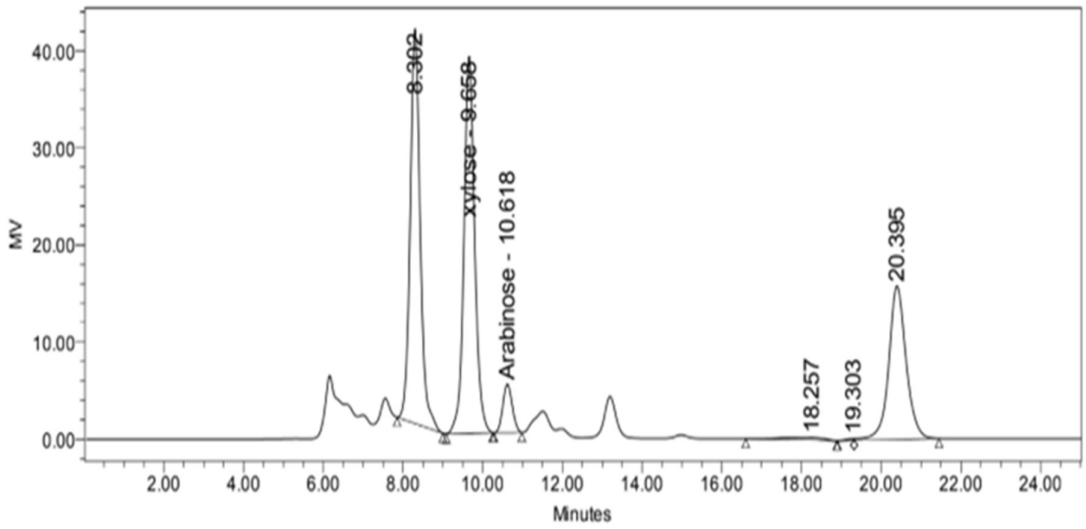

Figure S4. HPLC chromatogram showing peak of residual sugar (Xylose RT 9.6 min) and ethanol (RT 20.3 min) produced after 72 h of fermentation of mixed biomass (paddy straw and jute) with *S. cerevisiae* JRC6.

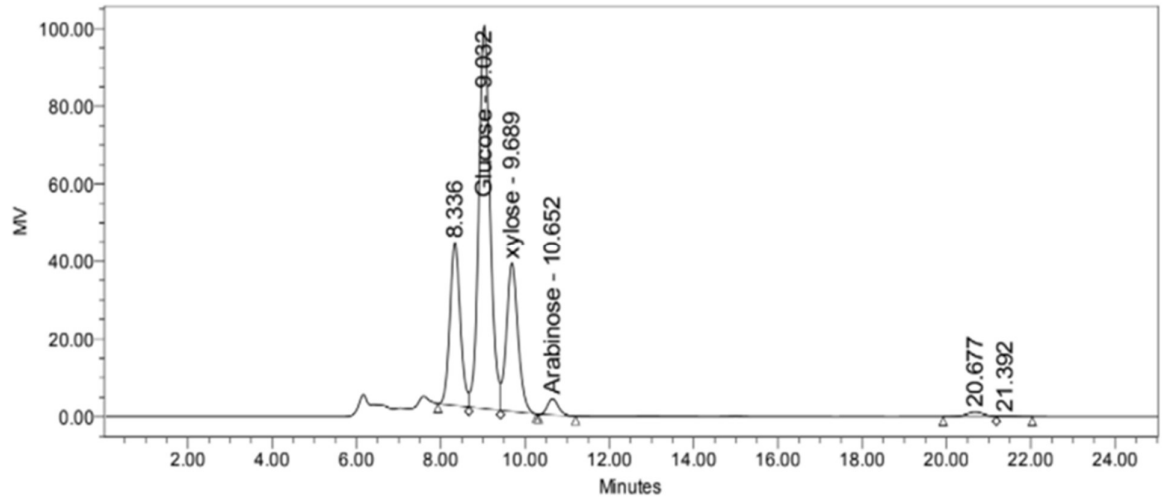

Figure S5. HPLC chromatogram showing peaks of sugar present in the saccharified hydrolysate of jute biomass before fermentation with LAB i.e., *Lactobacillus plantarum* LP-9.

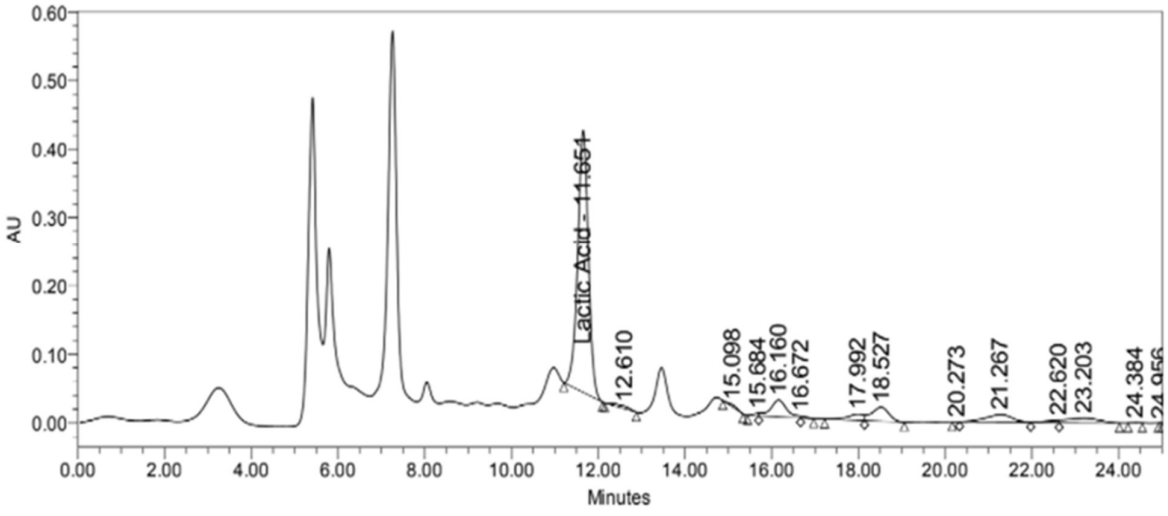

Figure S6. HPLC chromatogram showing peak of lactic acid (RT 11.6 min) after fermentation of saccharified hydrolysate of jute biomass.

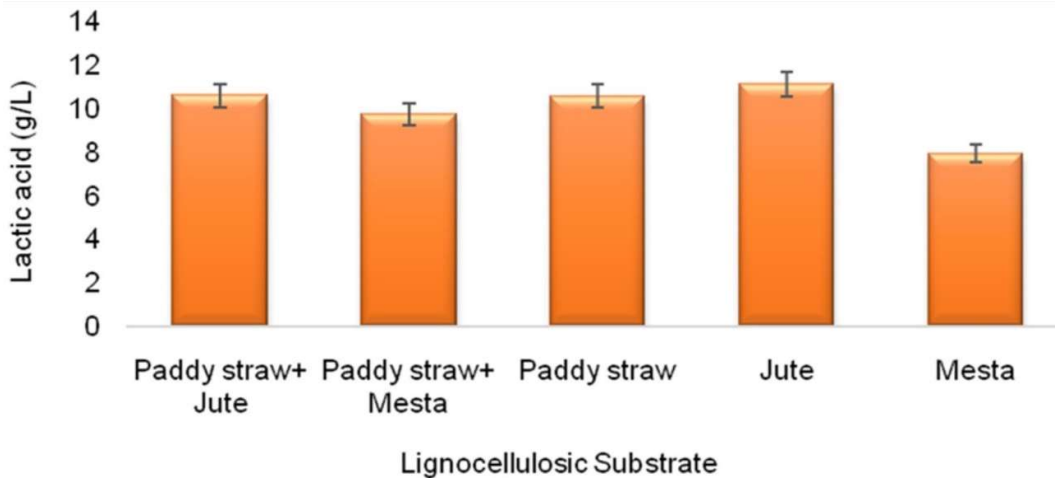

Figure S7. Lactic acid production (g/L) from saccharified individual and mixed substrate using *Lactobacillus plantarum* LP-9. Error bars represents SEM.

Table S1. Total reducing sugar released (g/L) after enzymatic saccharification of alkali pretreated mixed biomass (Paddy straw: Jute/ Mesta) in 3:1 ratio with varying substrate loading (%) at different time interval.

Substrate	**Paddy Straw: Jute**			**Paddy Straw: Jute**			**Paddy Straw: Jute**			**CD**
loading		**(1:1)**			**(2:1)**			**(3:1)**		
(%)	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	
5	5.13	6.97	8.08	7.25	8.61	10.42	9.49	11.96	14.05	1.137
	± 0.01	± 0.04	± 0.62	± 0.02	± 0.22	± 0.10	± 0.50	± 0.37	± 0.62	
10	21.29	25.86	30.34	23.61	29.43	33.32	28.44	36.70	41.50	4.486
	± 0.62	± 0.81	± 1.81	± 1.06	± 1.41	± 2.10	± 1.98	± 2.46	± 1.26	
Substrate	**Paddy Straw: Mesta**			**Paddy Straw: Mesta**			**Paddy Straw: Mesta**			
loading		**(1:1)**			**(2:1)**			**(3:1)**		
(%)	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	
5	4.04	4.28	6.26	6.58	7.71	9.85	10.29	11.70	12.73	0.848
	± 0.00	± 0.12	± 0.32	± 0.02	± 0.11	± 0.22	± 0.20	± 0.48	± 0.41	
10	22.00	26.79	29.88	24.50	28.16	32.73	27.56	34.90	38.50	3.142
	± 0.60	± 0.22	± 1.26	± 0.57	± 0.62	± 1.43	± 1.68	± 1.70	± 2.01	

All the experiments were performed with 3 replicates and their mean values tabulated. All the data obtained from the experiments were statistically analysed using the Three-Way ANOVA through GRAPHPAD PRISM 8.0 program (Microsoft Excel package, USA). Statistical significance was performed at $P = 0.05$ level where variances of Three-Way interaction was found to be non-significant. However, two-way interaction between substrate loading and substrate ratio, and substrate loading and time were found significant at 0.05% confidence interval.

Table S2. Ethanol production potential of various saccharified lignocellulosic biomasses as reported by other researchers

Substrate	Ethanol yield (g/L)	Reference
Cotton gin and paper sludge	6.75	(Shen and Agblevor, 2010)
Rice hull and cotton stalk	10.34	(Imamoglu and Sukan, 2014)
Coffee husk, cassava stem and coconut coir	9.50	(Nguyen et al. 2017)
Vetiver grass	6.00	(Subsamran et al. 2019)
Mesta biomass	4.10	(Lavanya et al. 2019)
Jute biomass	7.55	(Singh et al. 2020)
Paddy straw and jute	8.39	Present study

Table S3. Fatty acid profile of lipid extracted from mixed saccharified biomass (Paddy straw and jute) using oleaginous yeast *T. mycotoxinivorans* S2.

Component	Relative Percentage (%)
Palmitic acid $C_{16:0}$	15.78
Palmitoleic acid $C_{16:1}$	1.59
Oleic acid $C_{18:1}$	69.72
Linoleic acid $C_{18:2}$	4.57
α-Linolenic acid $C_{18:3}$	1.14
Lignoceric acid $C_{24:0}$	7.19

Table S4. Lipid production potential of different oleaginous yeast strains from different saccharified agroresidues.

Feedstock	Microbial strain	Lipid yield (g/L)	Reference
Rice straw hydrolysate	*T. fermentans*	11.50	(Haung et al. 2009)
Corn stover	*Trichosporon cutaneum* AS 2.571	7.60	(Hu et al. 2011)
Corn cobs residue	*T. cutaneum* ACCC20271	12.30	(Gao et al. 2014)
Douglas fir forest residue	*Mortierella isabellina* NRRL 1757	14.40	(Harde et al. 2016)
Paddy straw	*T. mycotoxinivorans* S2	7.32	(Sagia et al. 2020)
Paddy straw and jute	*T. mycotoxinivorans* S2	14.50	Present study

Table S5. Lactic acid production by different lactic acid bacteria from saccharified lignocellulosic biomass.

Substrate	Microorganism	Lactic Acid Yield (g/L)	Reference
Wheat straw hemicellulose	*Lactobacillus brevis* and *L. pentosus*	4.70 6.60	(Garde et al. 2002)
Sugarcane bagasse	*Lactococcus lactis IO-1*	10.85	(Laopaiboon et al. 2010)
Corn stover	*L. rhamnosus* and *L. brevis*	17.75	(Cui et al. 2011)
Cottonseed meal	*Sporolactobacillus inulinus YBS1-5*	3.70	(Bai et al. 2016)
Jute biomass	*L. plantarum* LP-9	11.08	Present study

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